compounds in which the oxygen potential is ≧ that of atmospheric oxygen, and an oxidation place for substances in which it is less than that of atmospheric oxygen. In the absence of some agreement as to the zero point the discussion is likely to be as confused in the future as it has been in the past.]

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Studies on Enzyme Action. XXII.—*Lipase* (IV)—*The Correlation of Synthetic and Hydrolytic Activity.*

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In the previous communication on this subject, in which the behaviour of Lipase towards ethereal salts generally was discussed, it has been argued that the enzyme is specially fitted to determine the hydrolysis of the insoluble, oily, glyceric salts of the higher fatty acids but is not suited to act in aqueous solutions: we expressed the opinion that interaction must be supposed to take place at and between surfaces separated only by a thin film of water at most—in other words, that water in excess is inimical to the occurrence of change. The results we advanced, in conjunction with those deduced from the study of other enzymes, notably urease, also led us to conclude that it is impossible to apply the laws of mass action directly to the interpretation of the changes effected by Lipase.

Previously we have directed our attention only to the hydrolytic activity of the enzyme: numerous observations are on record which prove that, whether of animal or vegetable origin, it can act reversibly but no comparative study of the two processes has been made hitherto in the case of fats.*

* (1) Kastle and Löwenhardt, 'Amer. Chem. Journ.,' vol. 24, p. 491. (2) Hanriot, 'Compt. Rend.,' vol. 132, p. 212 (1901). (3) Pottevin, *ibid.*, vol. 136, p. 1152 (1903); (4) 'Bull. Soc. Chim.,' III, vol. 35, p. 693 (1906). (5) Dietz, 'Zeit. Physiol. Chem.,' vol. 52,

In view of present ignorance of the manner in which fats are formed in the organism and the desirability of determining the extent to which their synthesis can be effected, under various conditions, we have carried out a series of parallel experiments to ascertain the limits within which the two opposing changes take place in presence of different proportions of the interacting substances and of water.

In the first series of synthetic experiments, 4·84 grm. of the fatty acids from olive oil (the amount equivalent to 5 grm. of the oil) was used, in each case, together with the quantity (0·53 grm.) of anhydrous glycerol that would be required if the whole of the fatty acid were to be converted into triglyceride.

The acid and glycerol were weighed into a 50-c.c. Jena glass flask together with 0·5 grm. of the enzyme preparation and 0·5 c.c. of toluene. The flasks were closed with rubber stoppers and kept slowly rotating in an incubator† maintained at 30° C. during the times stated. Alcohol was then added and the residual acid titrated with a normal solution of caustic soda. Each determination was made in duplicate and control experiments were carried out simultaneously with a preparation that had been boiled with water to destroy the activity of the enzymes. The results are given in the following table:—

Table I.—Synthesis of Fat from three Molecular Proportions of Acid to one of Glycerol.

Time	Acidity of control	Acidity of mixture containing enzyme		Percentage of acid combined
hours				
1	17·00	15·72	15·71	8·0
2	17·07	14·90	15·03	12·4
4	17·06	13·09	12·92	23·9
8	17·04	11·37	11·29	33·8
17	16·93	10·61	10·61	37·9
30	16·91	10·53	10·67	38·0
50	16·65	10·43	10·33	39·2
70	16·70	10·47	10·62	38·3

p. 279 (1907). (6) Hamsik, *ibid.*, vol. 59, p. 1 (1909). (7) Bradley, 'Journ. Biol. Chem.,' vol. 8, p. 251 (1910). (8) Taylor, 'Univ. California Pub. Path.,' vol. 1, p. 33 (1904); (9) 'Journ. Biol. Chem.,' vol. 2, p. 102 (1906). (10) Fokin, 'Chem. Rev. Fett-u.-Harz. Indust.,' vol. 13, p. 238 (1906). (11) Welter, 'Zeit. angew. Chemie,' vol. 24, p. 385 (1911). (12) Dunlap and Gilbert, 'Amer. Chem. Soc. Journ.,' vol. 33, p. 1787 (1911). (13) Kransz, 'Zeit. angew. Chemie,' vol. 24, p. 829 (1911). (14) Jalander, 'Biochem. Zeit.,' vol. 36, p. 435 (1911). (15) Bournot, *ibid.*, vol. 52, p. 172 (1913).

† That described in the previous communication ('Roy. Soc. Proc.,' B, vol. 86, p. 589). It may be noted that the figure there given is printed upside down.

To discover whether a true equilibrium had been reached or whether the action had ceased owing to the destruction of the enzyme, 0·5 grm. of enzyme was added to the system after the expiration of 24 hours and the mixture was titrated at the end of a second period of 24 hours. Experiments were also made in which 0·5 and 1 grm. of the enzyme were allowed to act during 48 hours before titrating the residual acid.

	Percentage of acid combined
0·5 grm. enzyme during 24 hours.....	37·4
0·5 48	37·7
1·0 48	33·6
0·5 24 Together with 0·5 grm. during a second period of 24 hours	34·9 35·3

The slightly lower activity observed in the experiments with 1 grm. of enzyme may have been due to the slight amount of water introduced with the preparation.

Further evidence that a true equilibrium had been reached was obtained on hydrolysing olive oil by the theoretical minimum amount of water, *i.e.* three molecular proportions to each molecular proportion of triglyceride or 5 grm. of oil and 0·53 grm. of water, quantities equivalent to those used in the synthetic experiments. As in the reverse case, the equilibrium was quickly reached and the acidity of the system was approximately the same as that observed in the experiments in the reverse direction.

Table II.—Hydrolysis of Fat by three Molecular Proportions of Water.

Time	Percentage of acid liberated
hours	
1	30·6
2	45·5
4	56·0
8	61·3
17	62·0
30	62·9
50	62·6
68	62·0

The addition of even a small amount of water influences the equilibrium to a marked extent and also has a retarding effect—to an increasing extent, moreover, as the amount of water is increased. This is shown in the following table, in which are recorded the results obtained by the synthetic action of 0·5 grm. of enzyme on mixtures of 4·84 grm. of fatty acid from

olive oil and 0·53 grm. of glycerol, together with from 0·31 to 3·1 grm. of water, *i.e.* from 3 to 30 molecules per molecule of glycerol.

Table III.—Synthesis of Fat in Presence of various Molecular Proportions of Water—showing Percentage of Acid combined.*

Time	No water	3 mols.	6 mols.	15 mols.	30 mols.
hours					
1	8·0	7·8	4·7		
2	12·4	12·0	7·3	3·6	
4	23·9	16·3	10·3		2·4
8	33·8	19·4	12·4	5·8	3·5
17	37·9	22·6	13·8	6·0	2·9
30	38·0	22·2		5·9	3·4
50	39·2	22·8		6·7	
70	38·3	21·5	15·2	6·8	3·9

Water also has a marked retarding effect on the rate at which the hydrolysis is effected, as shown in the following table, in which is given the percentage of acid formed on hydrolysing 5 grm. of olive oil in presence of from 3 to 24 molecular proportions of water per molecular proportion of glyceride. In these experiments, the difference between duplicate observations was somewhat greater than in the case of the synthetic experiments.

Table IV.—Hydrolysis of Fat in Presence of various Proportions of Water—showing Percentage of Acid liberated.

Time	3 mols.	6 mols.	9 mols.	15 mols.	24 mols.
hours					
1	30·6	27·2	19·4	13·1	9·6
2	45·5	40·6	27·4	19·1	15·9
4	56·0	61·7	46·7	36·2	23·2
8	61·3	73·1	56·5	49·9	36·7
17	62·0	77·0	74·8	63·0	55·2
30	62·9		83·6	75·7	66·1
50	62·6		85·2	80·2	77·2
70	62·0	78·7	84·8	82·6	81·2

It will be noticed that, in presence of 3, 6 and 9 molecular proportions of water, when equilibrium is reached, the acidity of the system is approximately the same as that observed in the corresponding synthetic series: but that when more water was present the effect on the enzyme was such that the equilibrium was not reached during the experiment.

* The results recorded are in all cases the means of duplicate experiments which differed by about 1 per cent. at most.

The effect of glycerol on the synthetic action is similar to that of water on the hydrolytic change, the equilibrium point being so shifted that more acid is removed from the system. Excess of glycerol retards the rate of change in a very noticeable manner. The amounts of acid which entered into combination in a mixture of 4·82 grm. of fatty acid and 1·06 grm. of glycerol, *i.e.* three molecular proportions of acid to two of glycerol, are shown in the following table.

Table V.—Synthesis of Fat in Presence of an excess of one Molecular Proportion of Glycerol.

Time	Acidity of system c.c. normal alkali		Percentage of acid combined
hours			
1	16·01	16·03	6·2
2	15·10	15·18	11·3
4	13·26	13·46	21·8
8	10·45	10·84	37·7
17	8·58	8·51	49·9
30	7·97	8·17	52·7
50	7·56	7·53	55·8
70	7·57	7·55	55·7

When three or more molecular proportions of glycerol are present to every three molecular proportions of acid, the retarding effect is so pronounced that no equilibrium point is reached within a reasonably convenient time, the acidity of the system falling slowly after 70 hours. Thus—

Glycerol, mol. props.	Acidity after 50 hours	Acidity after 70 hours
3	45·3	44·5
5	47·3	42·3
10	44·8	40·7

The effect of glycerol on hydrolysis is similar, as is shown in Table VI, in which is recorded the amount of acid liberated from 5 grm. of olive oil by 0·5 grm. enzyme and 0·31 c.c. water, in presence of 0·53 grm. of glycerol.

Table VI.—Hydrolysis of Fat in Presence of one Molecular Proportion of Glycerol.

Time.	Acidity of system in c.c. normal alkali.		Percentage of acid liberated.
hours.			
2	3·54	3·52	20·6
4	4·98	5·08	29·4
8	6·05	6·13	35·6
17	7·06	7·42	42·3
30	7·73	7·38	44·3
50	7·73		45·3
70	7·70	7·64	44·9

When more glycerol is present hydrolysis takes place to a reduced extent, and still proceeds slowly even after 70 hours.

Glycerol, molecular proportions.	Percentage of acid after 50 hours.	Percentage of acid after 70 hours.
2	29·8	30·9
4	11·0	12·6

The results of the experiments described are summarised in Graphs 1, 2 and 3, the synthetic observations in Graph 1, the hydrolytic in Graph 2, the parallel series of observations in the two opposite directions in Graph 3. The manner in which water affects both the rate of the change and the extent to which this takes place in one or the other direction is brought out in a very striking manner in these diagrams: it will be noticed especially how much less rapid is the approach, both from the hydrolytic and the synthetic side, to an equilibrium as the amount of water present is increased. Whilst the retardation of the hydrolytic change must be ascribed to a direct interference of the water, which presumably prevents the enzyme and the oil from coming into effective contact, the retardation of change in the opposite direction, especially the diminution of the extent to which synthesis takes place, must be ascribed rather to the withdrawal of glycerol from the system through its dissolution in the water: in this connexion, it is remarkable that synthesis is not entirely prevented even by the presence of thirty molecular proportions of water to one of glycerol, whilst in absence of an excess of water, an excess of glycerol beyond two molecular proportions has but little effect in increasing the proportion of fat synthesised.

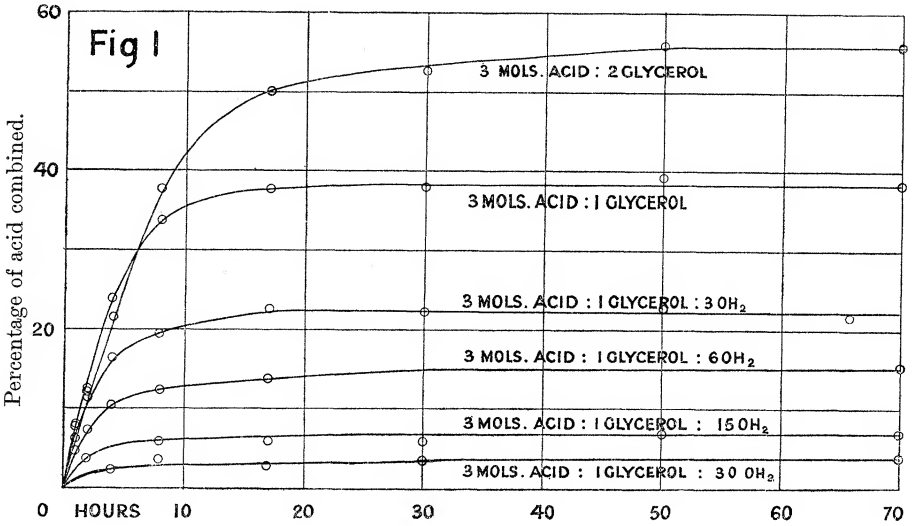
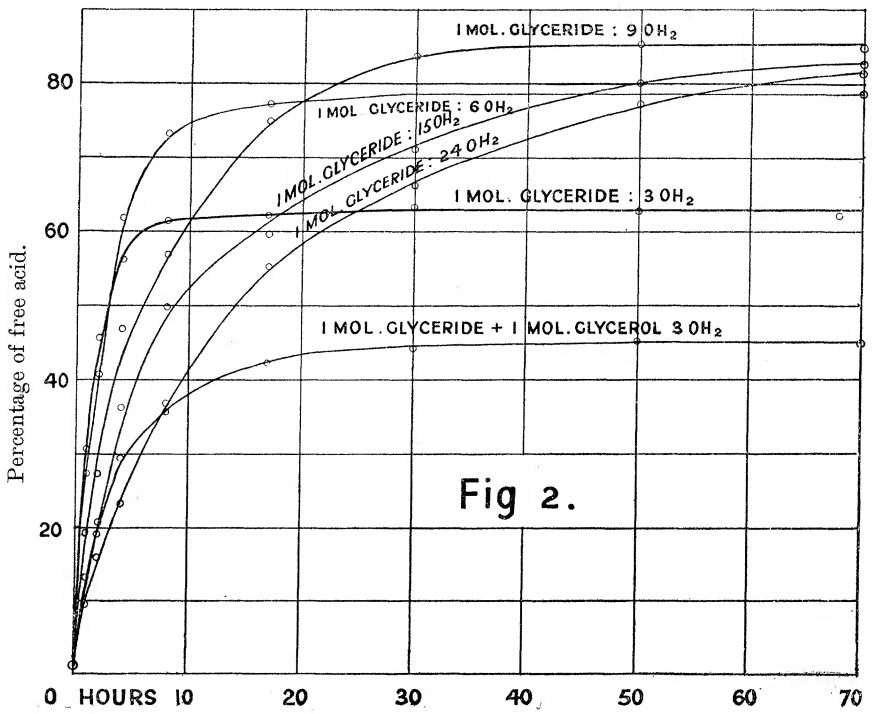


Fig 1
Percentage of acid combined.
60
40
20
0 HOURS 10 20 30 40 50 60 70
3 MOLS. ACID : 2 GLYCEROL
3 MOLS. ACID : 1 GLYCEROL
3 MOLS. ACID : 1 GLYCEROL : 3 OH2
3 MOLS. ACID : 1 GLYCEROL : 6 OH2
3 MOLS. ACID : 1 GLYCEROL : 15 OH2
3 MOLS. ACID : 1 GLYCEROL : 30 OH2


Percentage of free acid.
80
60
40
20
0 HOURS 10 20 30 40 50 60 70
1 MOL. GLYCERIDE : 9 OH2
1 MOL GLYCERIDE : 6 OH2
1 MOL. GLYCERIDE : 15 OH2
1 MOL. GLYCERIDE : 24 OH2
1 MOL. GLYCERIDE : 3 OH2
1 MOL. GLYCERIDE + 1 MOL. GLYCEROL 3 OH2
Fig 2.

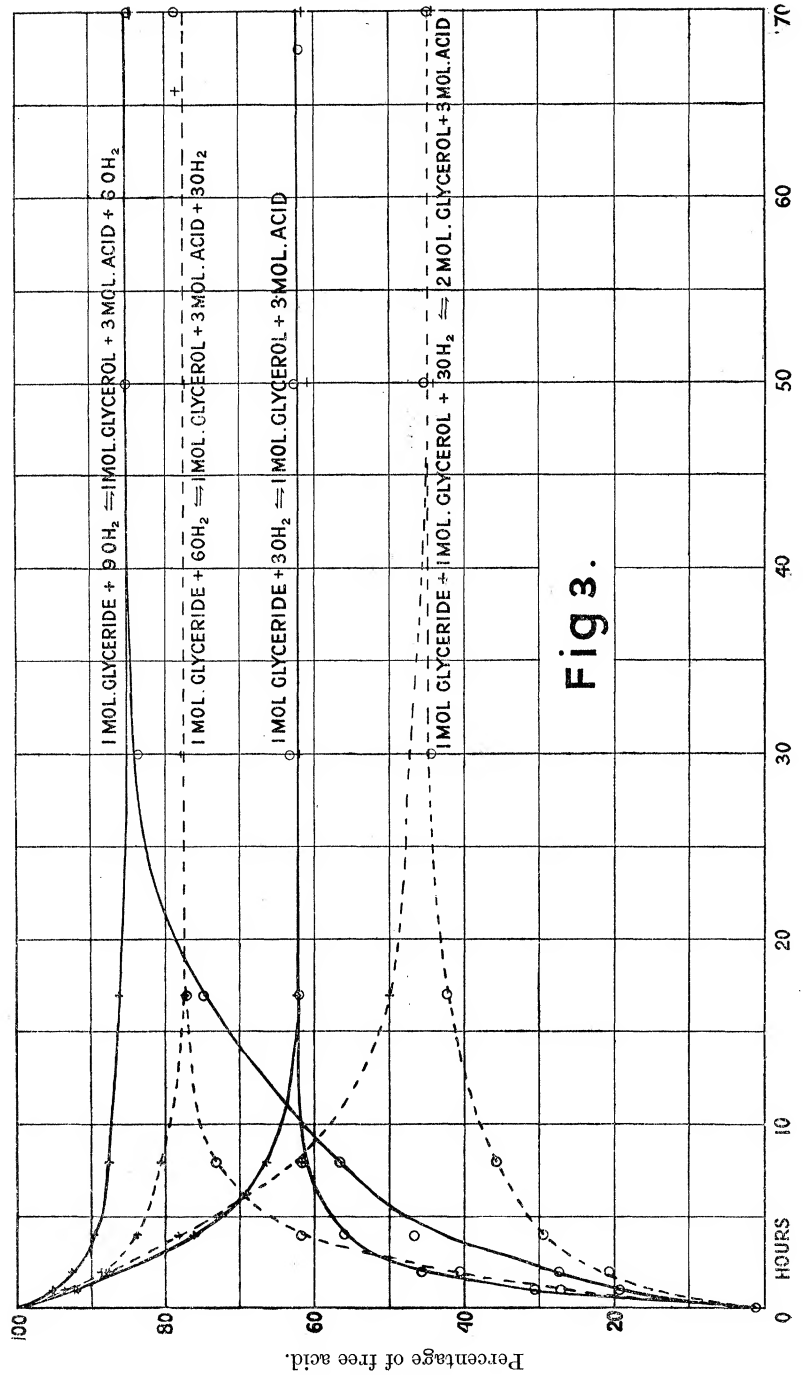

1 MOL. GLYCERIDE + 3 OH2 ⇌ 1 MOL. GLYCEROL + 3 MOL. ACID + 5 OH2
1 MOL. GLYCERIDE + 6 OH2 ⇌ 1 MOL. GLYCEROL + 3 MOL. ACID + 3 OH2
1 MOL GLYCERIDE + 3 OH2 ⇌ 1 MOL. GLYCEROL + 3 MOL. ACID
1 MOL GLYCERIDE + 1 MOL. GLYCEROL + 3 OH2 ⇌ 2 MOL. GLYCEROL + 3 MOL. ACID
Percentage of free acid.
HOURS
0
10
20
30
40
50
60
70
20
40
60
80
100

Fig 3.

In so far as our results can be brought into comparison with those of previous workers, they appear to be in harmony with their observations: but the activity of the enzyme we have had at our disposal, thanks to Tanaka's important discovery, appears to have been in excess of that used by others.

As it was obvious that if the limit reached in our synthetic experiments (about 40 per cent. when equivalents are used) were to be exceeded, the water produced in the interaction must be removed as it is formed, we endeavoured to secure this end by carrying out the synthesis *in vacuo* in a flask connected with drying apparatus. The results obtained have been uniformly unsatisfactory, inferior, in fact, to those obtained under ordinary conditions. Apparently, as pointed out by us previously, the intervention of a film of water is necessary at the interface of the system, where interaction takes place; if this be removed, action comes to an end.

In working with the Tanaka preparation, it is noticeable that the activity varies considerably, in a manner which is difficult to understand at first. On more than one occasion we have found that an enzyme which was quite active hydrolytically was inert when used as a synthetic agent with a mixture of acid and glycerol free from water: ultimately, this behaviour was traced to "overdrying," as on the addition of a very small amount of water the enzyme became active.

Enzyme which has been used and then recovered, by washing it free from oily matter by means of light petroleum, is found, as a rule, to be still active but usually less active than it was originally: the variable behaviour of such preparations is not surprising, however, in view of the colloid nature of the material and the effect which alterations in the state of aggregation and of surface conditions must have. It is noteworthy that the hydrolytic activity—in presence of a relatively large excess of water—of the enzyme is much more reduced by such treatment than the synthetic activity.

Nature of the Products of Change.—In order to ascertain whether the product of the synthetic action of Lipase is a nearly pure triglyceride like the natural fats and oils, the amount of glycerol uncombined in each experiment of the first series was determined, following the directions given by Lewkowitsch.

After titration, the contents of each flask was washed into an evaporating basin, boiled to expel most of the alcohol and then just acidified by sulphuric acid. After heating the liquid to the boiling point, the solution of glycerol was filtered off and the fatty acids and enzyme on the filter were then well washed with hot water: the filtrate was purified by addition of a solution of basic lead acetate and the glycerol estimated in the clear filtrate by Hehner's method (oxidation by an acid solution of potassium bichromate).

The method is probably one which is affected with a considerable error, so that the results have only qualitative significance.

Table VII.

Time	Glycerol found in			Percentage of acid combined	Mols. of acid combined per mol. glycerol
	Blank series*	Series A	Series B		
hours	grm.	grm.	grm.		
1	0·51	0·08	—	8·1	1·6
2	0·52	0·11	0·11	12·4	1·8
4	0·55	0·18	0·19	23·9	2·05
8	0·545	0·27	0·28	33·8	2·0
17	0·495	0·28	0·28	37·9	2·15
30	0·535	0·295	0·28	38·0	2·1
50	0·495	0·28	0·305	39·2	2·1
70	0·515	0·305	—	38·3	2·0

* The amount found should be about 0·53 grm.

In the same manner, estimations were made of the amount of glycerol liberated on hydrolysing olive oil by the enzyme in presence of 24 molecular proportions of water. It was found that, at first, the acids liberated were slightly in excess of the glycerol, an indication that a small quantity of a lower glyceride was formed: but as the action continued, the whole molecule was hydrolysed.

Table VIII.

Time	Glycerol found		Percentage of acid combined		Mols. of acid liberated per mol. glycerol
	Series A	Series B	Series A	Series B	
hours	grm.				
1	0·055	0·045	8·8	8·5	2·8
2	0·075	0·065	15·9	13·8	3·4
8	0·165	0·165	36·2	34·9	3·4
17	0·275	0·25	56·2	53·0	3·3
30	0·325	0·33	65·2	64·4	3·1
50	0·41	0·395	77·5	76·3	3·0
70	0·43	0·425	80·8	80·9	3·0

The amount of glycerol liberated, however, is less in proportion to the acid when the hydrolysis is brought about by a small proportion of water, showing that under these conditions mono- and di-glycerides are produced to a greater extent.

Thus, on hydrolysing 5 grm. of oil by 0·31 c.c. of water :—

Time	Percentage of acid liberated	Percentage of glycerol liberated	Mols. acid per mol. glycerol
hours			
1	31·0	24·5	3·8
2	45·6	36·8	3·7
8	61·8	46·3	4·0

Conversely, when the synthesis is effected in presence of water, less glycerol is combined than when no water is added; that is to say, the glycerides formed are more saturated. Thus, in presence of 0·62 c.c., *i.e.*, 6 molecules of water :—

Time	Percentage of acid combined	Percentage of glycerol combined	Mols. acid per mol. glycerol
hours			
2	7·7	12·0	1·9
8	11·9	15·0	2·4
70	15·2	18·5	2·5

An excess of glycerol not only alters the equilibrium so that a greater proportion of acid is combined but also influences the nature of the product, which then contains a smaller proportion of acid : thus the composition of the product of the interaction of two molecular proportions of glycerol and three molecular proportions of acid was found to be as follows :—

Table IX.

Time	Percentage of acid combined	Percentage of glycerol combined	Mols. acid combined per mol. glycerol
hours			
1	6·3	7·1	1·3
2	11·1	9·5	1·8
4	21·2	17·0	1·9
8	38·2	30·2	1·9
17	49·7	41·0	1·8
50	55·7	45·4	1·8

From these results, it is not improbable that the main product is a diglyceride : in other words, that, as is to be expected, the two primary hydroxyl groups of glycerol are first affected.

Some of the product of the interaction of the acids from olive oil with an excess of glycerol was isolated by evaporating off the alcohol after neutralising the unchanged acid and extracting the soap solution with ether. About

18 grm. of a pale yellow oil was thus obtained, which became turbid on standing, slowly clearing again on heating to 30° C. The saponification value of this oil was 183·5, that of the olive oil used being 191·7; on acetylation the saponification number was increased to 248·7, the "acetyl value" being 78·6. These data favour the assumption that the oil contained a high proportion of diolein. It is obvious, however, that no final conclusion is possible until experiments have been made with definite acids and the products have been isolated and characterised.

In view of our results, we venture to call attention to several directions in which the fats now deserve renewed attention.

Our knowledge of the manner in which they are absorbed and utilised under vital conditions is at present very vague in character and much of the evidence on which reliance is placed appears to be open to question. It is generally believed that, when ingested, fat is rapidly hydrolysed, under the influence of the pancreatic secretion and that derived from certain tracts of the intestine, this change being regarded as a necessary preliminary to its passage through the walls of the villi prior to entry into the circulatory system. Lipase appears to be widely distributed throughout the organism.

Apparently, whenever fat is to be transferred across cell membranes, it is hydrolysed: assisted by the emulsifying influence of the biliary fluid, the fatty acid that is liberated during digestion of fatty food can penetrate tissues that are impermeable to the fat but it is held that on entry into the villi the fatty acids are rapidly re-associated with glycerol and pass into the lacteals as fat. In fact, all fat that is stored is supposed to be fat that has been reconstituted from fatty acids. In the normal heart and other tissues, however, the fatty acids are not present as glycerides but apparently are combined in such a way that their histological behaviour is different from that of fats—the discriminative staining agents being without effect in such cases.

If the vital mechanism be such that only fatty acids can pass through, it is clear that in presence of lipase fats would undergo complete hydrolysis readily, under natural conditions, if the acids were removed as they were liberated, as reversal would be prevented.

Our observations appear to show that hydrolysis would be most rapid in presence of a minimum amount of water; they therefore favour the conclusion that conditions which would tend to reduce the concentration of the cell fluid would promote the conservation of fat — a conclusion which is perhaps applicable in explanation of the obesity which apparently is a frequent consequence of the indulgence in large quantities of weak alcoholic fluids such as Lager beer.

But in view of our observation that under 40 per cent. of fatty acid is convertible into fat, even when no water is present, it is difficult to understand how the fatty acids are completely reconverted unless there be some mechanism whereby the fat is separated from the fatty acid as it is formed—or some means by which the acid is held in abeyance until it is required. May it not be that the clue is afforded by the observations above referred to with reference to the presence of fat in the tissues in a cryptic form? Lipase apparently is a "carboxylase" which has the power of determining the hydrolysis of the ethereal salts of all the very weak carboxylic acids and, within limits, is more effective the less soluble the acid and the alcohol from which the salt is derived; presumably, the argument applies equally to the synthetic activity of the enzyme. It is therefore probable that, under the influence of lipase, fatty acid may become associated with hydroxylic centres in the protoplasmic complex and that such withdrawal may be the cause of its cryptic existence in muscular tissue.

The effect on health of an absence of fat from the diet, to which Arctic travellers have called attention, is noteworthy from this point of view. Stefánsson, in his recent book 'My Life with the Eskimo,'* states that the symptoms that result from a diet of lean meat are practically those of starvation; during the winter period, even when gorged with caribou meat free from fat, he and his party felt continually hungry; the dogs, though they got more meat than dogs usually get, were nothing but skin and bones. Previously, when they had lived practically on oil alone, taking a teacupful of oil a day, there were no symptoms of hunger; they grew each day sleepier and more slovenly, he says, but at the end of their meal of long-haired caribou skin (to give bulk) and oil felt satisfied and at ease.

On the assumption that fat is not always laid down as such but frequently reconstructed *in situ*, the presence of glycerol in the necessary amount at the seats of synthesis has to be accounted for. Owing to the solubility of this substance, it cannot well be supposed that, when fat is hydrolysed, the fatty acid and glycerol always remain together in the required proportions: it is more probable that the glycerol becomes separated from the acid to a greater or less extent and that the deficit is derived from carbohydrate: it is on this account, at least in part, perhaps, that it is desirable that a certain minimum ratio should be preserved between fat and carbohydrate in our food.

We are indebted to the Hull Oil Manufacturing Company, Ltd., for having placed at our disposal Indian castor seed of recent growth for the purpose of this inquiry.

* Macmillan and Co., London, 1913, pp. 140–141.

[*Note added June* 18.—In a communication which came to our notice only when the work we have described was completed Bournot (15) has called attention to the activity of the lipase present in the seeds of *Chelidonium majus*, the common Celandine, a papaveraceous plant. Having been able, through the courtesy of Messrs. Parke, Davis and Co., to obtain a sample of the seed, we have contrasted its activity with that of our *Ricinus* lipase and have confirmed Bournot's statement that it is not necessary to treat the seed with acid to render it active.

According to Bournot, *Chelidonium* lipase differs from *Ricinus* lipase in being most active in a neutral medium, even N/50 acid having an inhibitory effect. But as is shown in Part II, when once liberated from its zymogen *Ricinus* lipase is also sensitive to acid: in our experience, it has maximum activity when the acidity does not exceed that of oleic acid.

The enzymes from the two sources both hydrolyse and synthesise glyceric oleate with about the same ease and give rise to mixtures similar in composition at the equilibrium point. But weight for weight, the Tanaka *Ricinus* preparation is less active than *Chelidonium* seed (free from oil) in effecting the synthesis of isoprimary butylic oleate. Thus in an experiment in which 41 per cent. of the acid was combined by the agency of the *Ricinus* enzyme, about 80 per cent was etherified by *Chelidonium* seed. Apparently, the alcohol has a specially marked effect on the *Ricinus* preparation, as olive oil is hydrolysed only to a small extent in presence of a molecular proportion of isobutylic alcohol to one of the oleate.

Similarly, on hydrolysing isobutylic oleate, whereas, in presence of a single molecular proportion of water, 9·3 per cent. of change was effected in 17 hours by the *Chelidonium* enzyme, the *Ricinus* preparation caused only 2·4 per cent. of change. The difference was less marked on using 10 times as much water, as 16·7 per cent. was hydrolysed by the one and 13·0 per cent. by the other "enzyme": in this case, the effect of the alcohol was reduced apparently by the presence of the excess of water.

In our opinion, such differences as are observed are to be regarded, provisionally at all events, as consequences of differences in the "condition" of the enzyme in the different seeds. At present, as it is impossible to arrive at any estimate of the "concentration" of an enzyme or to allow for differences in its distribution, we cannot well make any valid comparison of the enzymes of like function derived from different sources.]